Asymmetric reproductive character displacement in male aggregation behaviour

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Reproductive character displacement—the evolution of traits that minimize reproductive interactions between species—can promote striking divergence in male signals or female mate preferences between populations that do and do not occur with heterospecifics. However, reproductive character displacement can affect other aspects of mating behaviour. Indeed, avoidance of heterospecific interactions might contribute to spatial (or temporal) aggregation of conspecifics. We examined this possibility in two species of hybridizing spadefoot toad (genus Spea). We found that in Spea bombifrons sympatric males were more likely than allopatric males to associate with calling males. Moreover, contrary to allopatric males, sympatric S. bombifrons males preferentially associated with conspecific male calls. By contrast, Spea multiplicata showed no differences between sympathy and allopatry in likelihood to associate with calling males. Further, sympatric and allopatric males did not differ in preference for conspecifics. However, allopatric S. multiplicata were more variable than sympatric males in their responses. Thus, in S. multiplicata, character displacement may have refined pre-existing aggregation behaviour. Our results suggest that heterospecific interactions can foster aggregative behaviour that might ultimately contribute to clustering of conspecifics. Such clustering can generate spatial or temporal segregation of reproductive activities among species and ultimately promote reproductive isolation.

Keywords: speciation; reinforcement; hotspot hypothesis; hotshot hypothesis; sexual selection; multi-species assemblages

1. INTRODUCTION

Reproductive character displacement is the process by which selection drives the evolution of traits that minimize reproductive interactions between species [1–4]. Reproductive character displacement stems from selection to avoid hybridization between species or to minimize reproductive interference between species that may search for and signal to prospective mates at the same times or locations [3–6]. When reproductive character displacement derives from selection to avoid hybridization, the process is considered ‘reinforcement’ [3,4,7–9]. Most studies of reproductive character displacement, including those of reinforcement, focus on the evolution of traits that are involved directly in mate attraction and reproduction [4,7]. Indeed, mating signals, mate preferences, morphology or reproductive physiology are often considered the targets of selection to minimize reproductive interactions between species [2,4].

Yet reproductive character displacement can also unfold in characters associated with the timing or location of mating [10–13]. In particular, different species might partition where or when they mate in order to minimize reproductive interactions with heterospecifics (reviewed in [5]). Individuals of a given species might become associated with a particular habitat or time of reproduction that differs from that of heterospecifics if doing so minimizes risk of heterospecific interactions (e.g. [14]). Likewise, individuals might actively associate with conspecifics if doing so facilitates identifying conspecific mates. For example, females might favour male aggregative behaviour if, in avoiding heterospecifics, females preferentially assess males that are members of conspecific groups. As a consequence of such behaviour, species may become non-randomly distributed in the timing or location of reproduction.

That species are often clustered in time or space with conspecifics for reproduction has been observed [15–22]. However, whether such behaviour stems from aggregation behaviour that has evolved in response to reproductive interactions with heterospecifics per se is often unclear. Individuals of different species may be independently drawn to particular times or places for reproduction, and this may be so for reasons other than to avoid heterospecifics. For example, species may differ in the location and timing of reproduction owing to different physiological requirements (e.g. temperature tolerance) [5].

Moreover, if conspecifics do display aggregative behaviour, they may do so for reasons other than to avoid heterospecifics. Indeed, conspecifics may cluster as a consequence of intraspecific competition for mates. For example, males might aggregate with others to maximize reproductive success by associating with attractive males (i.e. the ‘hotshot’ hypothesis) or by assembling in areas where females themselves cluster (i.e. the ‘hotspot’ hypothesis; reviewed in [15,23]). In multi-species contexts, such clustering within species could generate patterns of over-dispersion among species in the location or timing of reproduction. Thus, segregation of species in the timing or location of reproduction, which appears to

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Received 11 October 2010
Accepted 29 November 2010

doi:10.1098/rspb.2010.2196
Published online 22 December 2010

Proc. R. Soc. B (2011) 278, 2348–2354

Published online

2348

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2. MATERIAL AND METHODS

(a) Study system

We used the congeneric spadefoot toads *S. multiplicata* and *S. bombifrons* as our study system. These two species co-occur and risk hybridizing in the southwestern USA. Although hybridization between the species is potentially beneficial in some environments [24], hybridization can be costly: hybrid males are potentially sterile and hybrid females are only partially fecund [25,26]. Consequently, reproductive character displacement has promoted the evolution of female preferences and possibly male calls that facilitate mating with conspecifics [24,27,28].

Males might also have evolved expression of spatial differences in calling behaviour that potentially facilitates species recognition or minimizes interference of calls between the two species. In sympatry, *S. bombifrons* males call from sitting positions along the perimeter of breeding ponds, whereas *S. multiplicata* males tend to call from floating positions in the pond (K. Pfennig 1995–2010, personal observation). Indeed, *S. multiplicata* is significantly more clustered than expected by chance [29]. Such aggregation appears to result, in part, from males being attracted to the calls of conspecifics [29]. Whether spatial clustering of conspecifics or the differences between the species in calling position have arisen in sympathy (as opposed to also being expressed in allopatry) is unknown. However, in our study sites where *S. multiplicata* but not *S. bombifrons*, is found, *S. multiplicata* males still cluster. By contrast, in a population where *S. bombifrons* occurs without *S. multiplicata*, males were observed throughout the pond (K. Pfennig 2004, personal observation). These patterns are anecdotal, and provide mixed support for the possibility that males aggregate to minimize interactions with heterospecifics. For *S. multiplicata*, association behaviour may stem from within-species competition for mates: males aggregate with conspecific call stimuli that are attractive to females, presumably because doing so enhances the likelihood of encountering females [29].

Whether males aggregate to minimize interactions with heterospecifics or to intercept females, or to other males, are not mutually exclusive hypotheses. Nevertheless, disentangling which processes account for aggregative mating behaviour has important implications for understanding (i) when and how spatial and temporal aggregation evolves, and (ii) how such behaviour might minimize reproductive interactions with heterospecifics and possibly even contribute to reproductive isolation of species.

To evaluate whether male aggregation behaviour in spadefoots has evolved in response to selection to minimize reproductive interactions with heterospecifics, we measured male association behaviour with conspecifics for sympatric and allopatric males. We predicted that sympatric males of both *S. multiplicata* and *S. bombifrons* should be more likely than allopatric males to aggregate with conspecifics.

(b) Experimental design

We collected *S. multiplicata* and *S. bombifrons* males from allopatric and sympatric populations in Arizona, Colorado, New Mexico and Texas, USA. All toads were returned to the University of North Carolina at Chapel Hill, where they were fed nutrient-dusted crickets ad libitum.

Males in reproductive condition (as indicated by the presence of nuptial pads) were tested for their aggregation behaviour using previous methods [29]. We briefly summarize these methods below. Because our methods were nearly identical to the previous study, we pooled our data with those from Pfennig et al. [29]. In particular, Pfennig et al. [29] tested 26 sympatric *S. bombifrons*, 7 sympatric *S. multiplicata* and 21 allopatric *S. multiplicata* for their association with conspecific versus heterospecific calls; whereas in 2008 we tested 6 sympatric *S. bombifrons*, 45 sympatric *S. multiplicata*, 13 allopatric *S. bombifrons* and 47 allopatric *S. multiplicata*. We found no significant difference between males tested previously and those tested subsequently in terms of time spent with the conspecific stimulus minus the heterospecific stimulus (details of this response measure are below; 1999 versus 2008: for sympatric *S. bombifrons*, Wilcoxon normal approximation $Z = 1.56$, $p = 0.12$; sympatric *S. multiplicata*, Wilcoxon normal approximation $Z = -0.97$, $p = 0.33$; allopatric *S. multiplicata*, Wilcoxon normal approximation $Z = 1.46$, $p = 0.14$).

Pooling the data from the different experiments increased the number of populations from which males were sampled, increased statistical power and provided the following overall sample sizes of males that were tested: 32 sympatric *S. bombifrons* males; 52 sympatric *S. multiplicata* males; 13 allopatric *S. bombifrons*; and 68 allopatric *S. multiplicata*. For each species and within each region (sympatry and allopatry), individuals from different populations were pooled for analysis. Pooling across multiple populations from within sympatry and allopatry enhances the confidence that any differences detected between sympathy and allopatry can be attributed to the presence of heterospecifics per se rather than to an unknown variable specific to a particular population.

For *S. multiplicata*, the allopatric males were from within a region where *S. bombifrons* is restricted to lower elevations than *S. multiplicata* (i.e. our allopatric *S. multiplicata* were allotopic) [30]. Although these allopatric populations occur within 20 km of sympatric populations, *S. multiplicata* female choice differs between them [27], indicating that
the populations are sufficiently divergent for alternative behaviours to arise between them.

We used a two-speaker phonotaxis test to determine whether males preferentially associate with calls of conspecific males. Males were initially placed on a 22 cm tall block in the centre of a wading pool filled with 20 cm of water. This central block was equidistant between two identical blocks set 180° and 1.5 m apart, which were placed against the sides of the pool. The side blocks each supported a speaker from which we broadcast either a series of *S. multiplicata* calls or a series of *S. bombifrons* calls antiphonally (see below). Additionally, we placed two more identical blocks at the sides of the pool at 90° from those supporting the speakers. The presence of these blocks served as a control set up in a similar manner to those holding the speakers, but where no stimuli were presented. These alternative locations provided the males with structures that they could use without approaching the stimuli (e.g. they were a place where males could exit the water or seek a substrate away from the centre of the pool).

Each male was initially placed in an opaque container for a 10 min acclimation period. We began playing the stimuli at the start of this period. After acclimation, the male was released and the stimuli continued to play. The male’s location in the pool was continuously recorded for 30 min by an observer in a separate room via an infrared camera–monitor system.

We considered a male as associating with a stimulus when he was within an area surrounding the speaker that was approximately rectangular in shape (it was flush with the circular side of the pool) and which measured 95 × 53 cm (at its deepest point). This area corresponded to approximately the distance that often separates males in natural situations (figure 1; K. Pfennig 1995–2010, personal observation). This area was marked off with clearly visible lines on the bottom of the pool. To ensure consistency and objectivity among observers, a male was designated as within the region of association with a given stimulus when any part of his body was on or over the line demarcating the association area.

The remainder of the pool outside of these association areas was designated as ‘neutral’. Because we were interested in whether males responded to the stimuli, and which stimulus (if any) they preferred, we did not distinguish between the time spent in proximity to the control blocks (the blocks set at the side of the pool 90° from those supporting the speakers) versus the time spent in the remainder of the pool outside of the association area with the stimuli. The very presence of the control blocks reduced the possibility that males would approach the speakers purely as a means to escape the centre of the pool. Thus, if males merely preferred the sides of the pool (as opposed to the centre), then we did not expect to find differences among the males in our response variables.

If a male did not leave the neutral area or if he did not spend greater than 60 s with either of the stimuli during the 30 min observation period, he was considered non-responsive. The 60 s cut-off was somewhat arbitrary, but it was identified on the basis of seven outlier males. Of these males, five were *S. multiplicata* (three were from sympatry and two were from allopatry), and two were *S. bombifrons* (one was from sympatry and one was from allopatry). These seven males spent, on average, 28 s total in association with one or both speakers (the range was from a total of 2 s to 58 s). From behavioural observations, these males appeared to be simply ‘passing through’ the association areas on their way to neutral areas of the pool. Eighteen additional males were designated non-responsive because they spent no time in association with either stimulus.

Male aggregative behaviour can be analysed at two levels: their proclivity to associate with other males (regardless of species) in the first place, and their preference for a particular stimulus, if any, given their tendency to associate with other males. We divided males into ‘responsive’ and ‘non-responsive’ categories so as to determine whether males differed in the likelihood of even approaching the stimuli. We compared the likelihood of responding to the stimuli between the allopatric and sympatric males for each species using contingency table analyses. Doing so allowed us to ascertain whether males from sympatry versus allopatry were equally likely to engage in association behaviour with calling males (regardless of the calling male’s species).

All males were presented with *S. multiplicata* versus *S. bombifrons* calls. To create the sound stimuli, we chose *S. multiplicata* and *S. bombifrons* calls at random from a set of male calls recorded at natural sympatric breeding aggregations [27]. Each *S. multiplicata* call was randomly paired with a *S. bombifrons* call to create eight pairs of *S. multiplicata* versus *S. bombifrons* calls. For each set of calls, the *S. multiplicata* call was repeated onto a single track at the average call rate for *S. multiplicata*, and the *S. bombifrons* call was repeated onto a second track at the average call rate for *S. bombifrons* [27].
Males were randomly assigned to eight groups corresponding to the presented call pairs. Each male within a group was therefore presented with the same stimuli pair, and each group was presented with a different stimuli pair. Thus, all males were presented with the same stimuli (S. multiplicata versus S. bombifrons calls) across the groups, but the calls used in making those stimuli differed between the groups. This procedure of using different representations of the same stimuli ensured that males were responding to the call stimuli per se rather than an uncontrolled variable in a single natural male recording [31]. We switched the stimuli between speakers after each male we tested to control for side biases and equalized the speakers for volume. No male was used more than once.

To determine whether males preferentially associated with the conspecific stimulus as opposed to the heterospecific stimulus, we tallied the total amount of time each male spent with the conspecific stimulus and the heterospecific stimulus. We then calculated the difference in time spent between the stimuli as the total time with the conspecific stimulus minus that with the heterospecific stimulus. We then compared the mean value of this difference with a null expectation of zero for sympatric and allopatric males of each species. If males preferentially associate with conspecific male calls, we expected this difference to exceed zero. We then compared this mean difference in time spent with the stimuli between sympatric and allopatric males of each species. If males have evolved preferential association with conspecifics to avoid interactions with heterospecifics, then the difference in time spent with conspecifics versus heterospecifics should be higher in sympatry than in allopatry. Because the data for these analyses did not meet parametric assumptions, we used non-parametric Wilcoxon tests.

3. RESULTS

When we evaluated whether males in sympatry versus allopatry were differentially responsive to calling males, we found different results for the two species. Sympatric S. bombifrons males were significantly more likely than allopatric males to spend at least 1 min in association with a call stimulus (likelihood ratio $\chi^2 = 4.33, p = 0.04$). Indeed, 2 out of 32 sympatric S. bombifrons were non-responsive during the observation period (see §2), whereas 4 out of 13 allopatric S. bombifrons were non-responsive. By contrast, sympatric and allopatric S. multiplicata were equally likely to spend at least 1 min in association with a call stimulus (likelihood ratio $\chi^2 = 0.01, p = 0.91$). In particular, 8 out of 52 sympatric S. multiplicata were non-responsive during the observation interval, whereas 11 out of 68 allopatric S. multiplicata were non-responsive.

Of the males that associated with the call stimuli, sympatric males of both species spent significantly more time in association with the conspecific call stimulus as opposed to the heterospecific call stimulus (S. bombifrons: mean difference time (seconds) spent with conspecific – heterospecific $(\pm$ s.e.$) = 293.17$ (105.96); Wilcoxon signed rank $= 113.5, p = 0.02$; S. multiplicata: mean difference time (seconds) spent with conspecific – heterospecific $(\pm$ s.e.$) = 356.77$ (104.99); Wilcoxon signed rank $= 255.0, p = 0.002$). By contrast, neither S. bombifrons nor S. multiplicata males from allopatry spent more time with the conspecific calls (S. bombifrons: mean difference time (seconds) spent with conspecific – heterospecific $(\pm$ s.e.$) = -290.22$ (345.60); Wilcoxon signed rank $= -5.5, p = 0.57$; S. multiplicata: mean difference time (seconds) spent with conspecific – heterospecific $(\pm$ s.e.$) = 162.81$ (128.75); Wilcoxon signed rank $= 206.0, p = 0.10$).

Contrasts between allopatry and sympatry revealed character displacement in male aggregation behaviour for S. bombifrons only. When we contrasted the mean difference in time with the conspecific stimulus versus the heterospecific stimulus, we found that S. bombifrons’s time spent with conspecifics versus heterospecifics was significantly greater in sympatry versus allopatry (Wilcoxon normal approximation $Z = -2.22, p = 0.03$; figure 2a). By contrast, for S. multiplicata, the mean difference in time spent with conspecific calls versus heterospecific calls did not differ significantly between
sympatry and allopatry (Wilcoxon normal approximation \( Z = 0.72, p = 0.47; \) figure 2b).

4. DISCUSSION

Conspecific males of many species cluster in time or space to seek and attract mates. Why this is so is not always clear. Males might aggregate as a consequence of within-species selection (e.g. imposed by competitors or predators) [15]. Alternatively they might cluster with conspecific males as a means to minimize reproductive interactions with heterospecifics [5]. Here, we experimentally evaluated the latter hypothesis. We predicted that, if such behaviour evolves to minimize interactions with heterospecifics, then sympatric males should be more likely than allopatric males to associate with conspecifics.

Our findings with \( S. \) bombifrons (but not \( S. \) multiplicata) were consistent with our prediction. Contrary to allopatric \( S. \) bombifrons, sympatric \( S. \) bombifrons were more likely to associate with a calling male stimulus (regardless of species), and they preferentially associated with the conspecific stimulus. These results indicate that aggregative behaviour, which could ultimately promote spatial (or temporal) segregation among species, can evolve via selection to minimize reproductive interactions with heterospecifics. Thus, the effects of reproductive character displacement are not limited to promoting divergence in morphology and mating signals.

That sympatric \( S. \) bombifrons males are more likely than allopatric males to associate with conspecifics does not necessarily enable us to identify the particular reason why heterospecifics might be avoided. Because \( S. \) bombifrons risk costly hybridization with \( S. \) multiplicata, selection to minimize such risk seems a likely explanation for male aggregative behaviour in sympatry. In other words, such behaviour may derive from reinforcement [2,4,7,8].

However, males might also avoid heterospecific interactions to minimize interference with their sexual signals. Moreover, if heterospecific males interact aggressively, they might experience selection to avoid each other by partitioning their reproductive activities spatially or temporally (via agonistic character displacement [13]). Indeed, males might even avoid heterospecifics to minimize interspecific resource competition (e.g. for limited oviposition sites), in which case divergence in aggregation behaviour could reflect ecological character displacement [3].

In spadefoots, divergence in male aggregative behaviour with conspecifics is probably driven by selection to minimize hybridization. Males do not defend oviposition sites, they do not provide resources to females and they forage away from the breeding ponds [32]. Ecological character displacement mediated by resource competition is therefore not a likely explanation for our results.

Moreover, although male–male aggression can generate selection to avoid heterospecifics, it does not fully explain why sympatric males should be attracted to conspecifics. Male–male aggression—whether between or within species—typically produces over-dispersion of males (e.g. as when males hold territories or defend sites used in sexual signalling). Indeed, in a recent review [13], the putative cases (and theoretical analysis) of character displacement driven by agonistic interactions involve territorial species that potentially experience selection to recognize heterospecific competitors. In our experiment, male aggression would have been manifest as either avoidance of the call stimuli (resulting in no responses being measured) or aggression towards the call stimuli. Neither behaviour was observed in the sympatric males. Moreover, as described above, \( S. \) are not territorial and male–male aggression is not pronounced in this genus. Indeed, \( S. \) multiplicata males are significantly more clustered than expected by chance [29] (see also figure 1).

Selection to minimize signal interference can also explain why males avoid heterospecifics. Yet, as with male–male aggression, selection to minimize signal interference between species does not necessarily explain why males become attracted to conspecifics (with whom they would also experience signal interference). Although further study of male aggression and signal interference is warranted (both male–male aggression and the resolution of signal interference within conspecific groups can take subtle forms), these explanations seem less able to account for preferential association with conspecif\(c\)ics in this particular system.

By contrast, minimizing hybridization risk can explain attraction to conspecifics if aggregative behaviour maximizes the chances of mating with conspecifics. This hypothesis assumes that male aggregative behaviour actually promotes spatio-temporal clustering of conspecif\(\)ics, and predicts that hybridization is reduced when male aggregation behaviour—and therefore spatial (or temporal) segregation of species—is more pronounced.

In spadefoots, hybridization frequency increases with decreasing pond size [25,33]. One explanation for this pattern is that, in smaller ponds, male density is higher and males are less able to segregate spatially, thereby contributing to hybridization [25]. A confounding factor, however, is that females potentially benefit from hybridization in small ponds: hybrid tadpoles develop faster, and \( S. \) bombifrons females preferentially hybridize to accrue this fitness benefit in small, shallow ponds [24]. Thus, the degree to which hybridization is driven by females engaging in adaptive hybridization versus a breakdown in spatial segregation of conspecifics and heterospecifics is unknown. Indeed, the two might not be mutually exclusive mechanisms for explaining hybridization.

The possibility that \( S. \) bombifrons has evolved aggregative behaviour in response to heterospecifics is striking, given the presence of other traits that minimize reproductive interactions between these species. In particular, male calls have diverged between sympathy and allopatry (presumably because of selection to avoid hybridization, acoustic interference or both), and sympatric females have evolved preferences that facilitate conspecific matings when doing so is beneficial [24,28]. That sympatric and allopatric males also have diverged in aggregation behaviour suggests that selection might favour the evolution of redundant mechanisms to minimize interactions with heterospecifics.

Moreover, redundant traits that minimize heterospecific interactions can arise via correlated evolution [2,34,35]. Indeed, female preferences used in recognizing conspecifics may also indirectly favour male aggregative behaviour if females make fewer errors when choosing.
among conspecific males that happen to cluster. In such a case, female mate choice passively favours aggregative males, but females could also actively favour male aggregative behaviour with conspecifics if females avoid areas where heterospecifics are present or preferentially assess males that are members of conspecific aggregations. Thus, the degree to which male aggregative behaviour is selectively favoured and expressed may depend on female mate choice patterns and their fitness consequences. Ironically, however, spatial segregation of conspecific males away from heterospecifics could mitigate against selection on females to adopt preferences for discriminating conspecifics from heterospecifics. Generally, understanding how male and female behaviour evolve in tandem is critical for evaluating how selection acts to minimize reproductive interactions between species.

In contrast with *S. bombifrons*, the results for *S. multiplicata* did not fully comport with the notion that males aggregate to avoid heterospecific interactions. Although sympatric *S. multiplicata* males preferentially associated with the conspecific stimulus whereas allopatric *S. multiplicata* did not, sympatric and allopatric *S. multiplicata* males did not differ in their likelihood of responding to the stimuli or in the mean difference in time spent with the conspecific versus heterospecific stimulus. Thus, sympatric and allopatric *S. multiplicata* did not show clear evidence of divergence in aggregative behaviour with conspecifics. One explanation for this pattern is gene flow between sympatric and allopatric *S. multiplicata* populations, which can be adjacent to one another (see §2 above). Yet gene flow is low between sympatry and allopatry [30], and is therefore unlikely to account for our findings. Instead, *S. multiplicata* males probably engage in aggregative behaviour owing to intraspecific factors such as competition for access to mates [29] or possibly predation.

That *S. multiplicata* males may aggregate with conspecifics for other reasons should not suggest that male aggregative behaviour does not also minimize interactions with heterospecifics. Male aggregative behaviour that arises in allopatry as a response to other factors could be maintained or further enhanced in sympatry by the added benefit of minimizing costly reproductive interactions with heterospecifics. Indeed, in *S. multiplicata*, male preference for aggregating with conspecifics may have become refined in sympatry: the range of variation in time difference with conspecifics was lower in sympatry relative to allopatry (figure 2b).

That *S. bombifrons* has undergone reproductive character displacement in aggregative behaviour, whereas *S. multiplicata* seemingly has not, illustrates that reproductive character displacement need not be similar for the two interacting species (reviewed in [36]). Differences between species in the evolution of behaviours that facilitate conspecific mate recognition (i.e. asymmetric character displacement [36]) can arise for a number of reasons. For example, if *S. multiplicata* already aggregated for other reasons before secondary contact, but *S. bombifrons* did not, then divergence would only be clearly detected for *S. bombifrons*. Additionally, whether one or both species undergo reproductive character displacement in a trait might depend on the strength of selection favouring divergence between them [36]. In spadefoots, selection to avoid hybridization may be weaker for *S. multiplicata* males than for *S. bombifrons* males because hybrid offspring of crosses between *S. multiplicata* males and *S. bombifrons* females do not suffer the same disadvantages that the reverse type of hybrid suffers [33]. Consequently, *S. bombifrons* males may experience stronger selection to engage in behaviour that fosters conspecific matings.

Most studies of reproductive character displacement, especially in animals, focus on mate preferences or mating signals as key components of reproductive isolation [2,4,7]. Our study suggests that spatial segregation of conspecifics and heterospecifics—mediated by male aggregative behaviour—might also contribute to reproductive isolation between species. If this is so, conspecific males should be more tightly aggregated in sympathy relative to allopatry, and spatial (or temporal) segregation of species should preclude hybridization. Moreover, because males are often deemed the less choosy sex, particularly in species lacking male parental care, the role of male behaviour in reproductive isolation is often ignored. The results presented here suggest that—at least in some species—male behaviour may also play a key role in species recognition, and in the structuring and timing of reproduction in multi-species assemblages.

We are grateful to H. Davis, K. Posey and L. Exline for assistance with animal care and observations. We also thank D. Pfennig, A. Moczek, C. Ledon-Rettig, D. Kikuchi, J. Santos and two anonymous reviewers for comments that improved the manuscript. This research was supported by a grant from the National Science Foundation and a National Institutes of Health Director’s New Innovator Award to K.S.P.

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